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REPORT

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## The combination of trastuzumab and pertuzumab administered at approved doses may delay development of trastuzumab resistance by additively enhancing antibody-dependent cell-mediated cytotoxicity

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### ABSTRACT

Although the recently concluded CLEOPATRA trial showed clinical benefits of combining trastuzumab and pertuzumab for treating HER2-positive metastatic breast cancer, trastuzumab monotherapy is still the mainstay in adjuvant settings. Since trastuzumab resistance occurs in over half of these cancers, we examined the mechanisms by which treatment of intrinsically trastuzumab-resistant and -sensitive tumors can benefit from the combination of these antibodies. F(ab')<sub>2</sub> of both trastuzumab and pertuzumab were generated and validated in order to separately analyze antibody-dependent cell-mediated cytotoxicity (ADCC)-based and direct biological effects of the antibodies. Compared to monotherapy, combination of the two antibodies at clinically permitted doses enhanced the recruitment of natural killer cells responsible for ADCC, and significantly delayed the outgrowth of xenografts from intrinsically trastuzumab-resistant JIMT-1 cells. Antibody dose-response curves of *in vitro* ADCC showed that antibody-mediated killing can be saturated, and the two antibodies exert an additive effect at sub-saturation doses. Thus, the additive effect *in vivo* indicates that therapeutic tissue levels likely do not saturate ADCC. Additionally, isobole studies with the *in vitro* trastuzumab-sensitive BT-474 cells showed that the direct biological effect of combined treatment is additive, and surpasses the maximum effect of either monotherapy. Our results suggest the combined therapy is expected to give results that are superior to monotherapy, whatever the type of HER2-positive tumor may be. The combination of both antibodies at maximum clinically approved doses should thus be administered to patients to recruit maximum ADCC and cause maximum direct biological growth inhibition.

**Abbreviations:** ADCC, Antibody-dependent cell-mediated cytotoxicity; DAPI, diamidino-2-phenylindole; EC<sub>50</sub>, half maximal effective concentration; F(ab')<sub>2</sub>, F(ab')<sub>2</sub>, bivalent antibody fragment of 2 Fab domains connected by disulfide bonds, lacking the Fc domain; FCS, fetal calf serum; HER2, human epidermal growth factor receptor 2, (a.k.a. ErbB2); MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NK, natural killer; SCID, severe combined immunodeficiency

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### Introduction

Overexpression of HER2 is found in different types of human malignancies, including 20–25% of breast cancers<sup>1,2</sup> and 17–22% of gastric cancers,<sup>3</sup> and it is associated with aggressive growth and poor clinical outcomes. The breakthrough finding that some anti-HER2 antibodies can inhibit the growth of cancer cells overexpressing HER2 on their surface led to the development of several antibodies against the extracellular domain of the protein.<sup>4</sup> Trastuzumab (Herceptin<sup>®</sup>), a humanized anti-HER2 monoclonal antibody, was the first antibody approved as a treatment for HER2-positive metastatic breast cancer,<sup>5</sup> and, due to its remarkable success, it is currently a first-line treatment. Its ability to inhibit *in vitro* and *in vivo* tumor growth<sup>6,7</sup> is attributed to internalization and down-regulation of cell surface HER2,<sup>8</sup> inhibition of the PI3K/Akt pathway,<sup>9</sup> cell cycle

arrest in G1, inhibition of angiogenesis<sup>10</sup> and antibody-dependent cell-mediated cytotoxicity (ADCC).<sup>11,12</sup>

Despite encouraging clinical results, about half of the HER2-positive cancers are primarily resistant to trastuzumab or become resistant during prolonged treatment.<sup>6,7</sup> Many potential mechanisms by which resistance to targeted antibody therapy may develop have been described, including steric hindrance by the extracellular matrix (ECM) proteins,<sup>13</sup> increased signaling from the insulin-like growth factor-I receptor,<sup>14</sup> constitutive activation PI3K/Akt pathways<sup>15</sup> or impaired ADCC response.<sup>16,17</sup>

We previously showed that HER2-positive JIMT-1 human breast cancer cells are intrinsically resistant to trastuzumab *in vitro* due to steric hindrance caused by ECM components, such as MUC4<sup>13</sup> and CD44-bound hyaluronic acid.<sup>18</sup> However,

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trastuzumab treatment can delay the outgrowth of JIMT-1 xenografts in severe combined immunodeficiency (SCID) mice, if treatment is started at the time of tumor inoculation, but not if treatment starts after the tumor has reached a few hundred mm<sup>3</sup>.<sup>12</sup> The antitumor effect is the result of ADCC mediated by trastuzumab, and cells that have little or no ECM, such as freshly trypsinized and injected tumor cells, as well as circulating and disseminated tumor cells,<sup>19</sup> are the primary targets.

Pertuzumab (Perjeta<sup>®</sup>), another humanized monoclonal antibody, targets the dimerization arm of HER2, which is distinct from the binding site of trastuzumab.<sup>20</sup> Through blocking HER2 dimerization with other HER (ErbB) family members, it inhibits downstream mitogenic signaling processes.<sup>20</sup> Because trastuzumab and pertuzumab bind to distinct epitopes on HER2, it has been hypothesized that a combination of the 2 agents might provide a more effective inhibition of tumor growth than either agent alone. In fact, combining 2 HER2-targeting antibodies against xenografted, in vitro trastuzumab-sensitive N87 gastric cancer cells synergistically decreased tumor growth, reciprocating the in vitro antiproliferative effect of the combination.<sup>20,21</sup> Since trastuzumab binds to HER2 close to the cell membrane, while pertuzumab binds to the dimerization arm, which is more distal from the membrane,<sup>20,22</sup> it is possible that, in tumors where ECM components interfere with trastuzumab binding, pertuzumab can still bind to HER2.

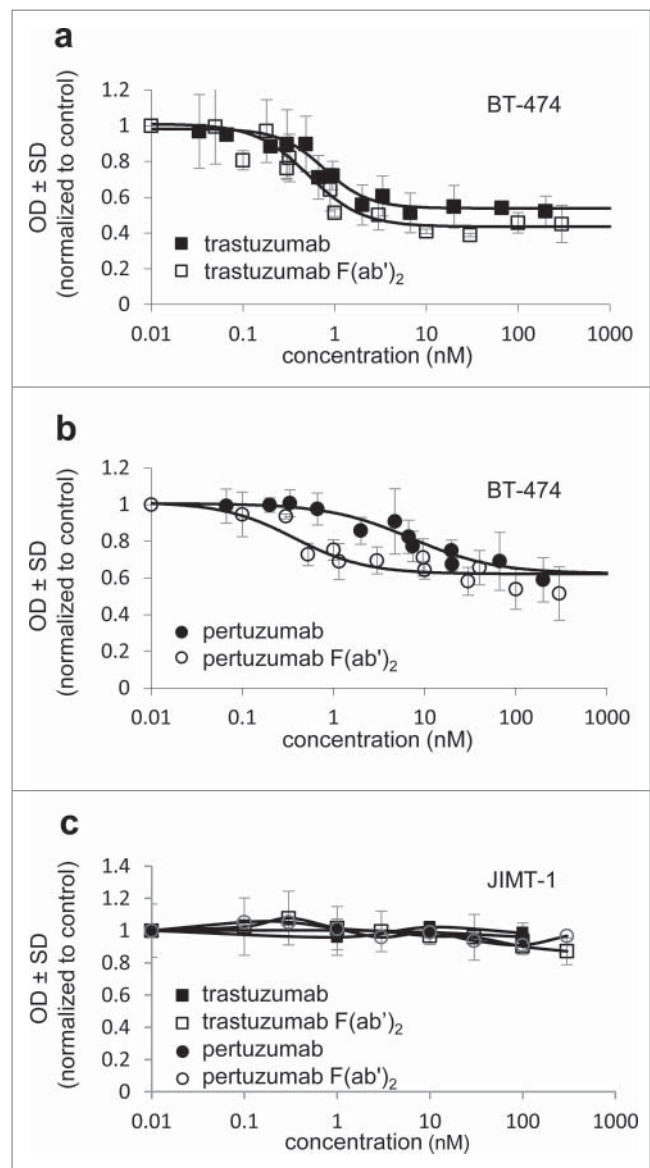
Treating HER2-positive malignancies with the combination of trastuzumab and pertuzumab has been explored in the preclinical setting,<sup>23,24</sup> and pertuzumab was approved for clinical use in combination with trastuzumab and docetaxel as neoadjuvant treatment for patients with HER2-positive locally advanced, inflammatory, or early-stage breast cancer in the USA<sup>25,26</sup> and metastatic or locally recurrent unresectable breast cancer in Europe.<sup>25,26</sup> Final overall survival analysis from the CLEOPATRA clinical trial showed that patients with metastatic HER2-positive breast cancer treated with this combination lived 15.7 months longer than those who received trastuzumab and chemotherapy alone, with a median overall survival of 56.5 vs. 40.8 months.<sup>27</sup> For postoperative adjuvant therapy of HER2-positive breast cancers, however, trastuzumab in combination with chemotherapy is still the current mainstay. The benefit of combination therapy is currently being further tested in the Phase 3 APHINTY trial (ClinicalTrials.gov identifier NCT01358877), to determine if the combination of pertuzumab and trastuzumab is better than trastuzumab alone, in terms of efficacy, for patients who have had surgery for HER2-positive breast cancer.

Because trastuzumab resistance in HER2-overexpressing breast cancers remains a central problem,<sup>28</sup> we aimed to explore the mechanisms by which the combination of trastuzumab and pertuzumab could provide benefits in treating HER2-positive tumors. We used the intrinsically trastuzumab-resistant breast cancer cell line JIMT-1 as in vitro ADCC target, and as xenograft tumors to show that, at tissue concentrations expected in the clinical setting, neither of the antibodies saturates ADCC, and consequently the combination of the 2 antibodies significantly delays tumor outgrowth by additively enhancing the recruitment of ADCC. In addition, isobole studies with the in vitro trastuzumab-sensitive BT-474 cells showed that the direct biological effect of combined treatment is additive, and surpasses the maximum effect of either monotherapy.

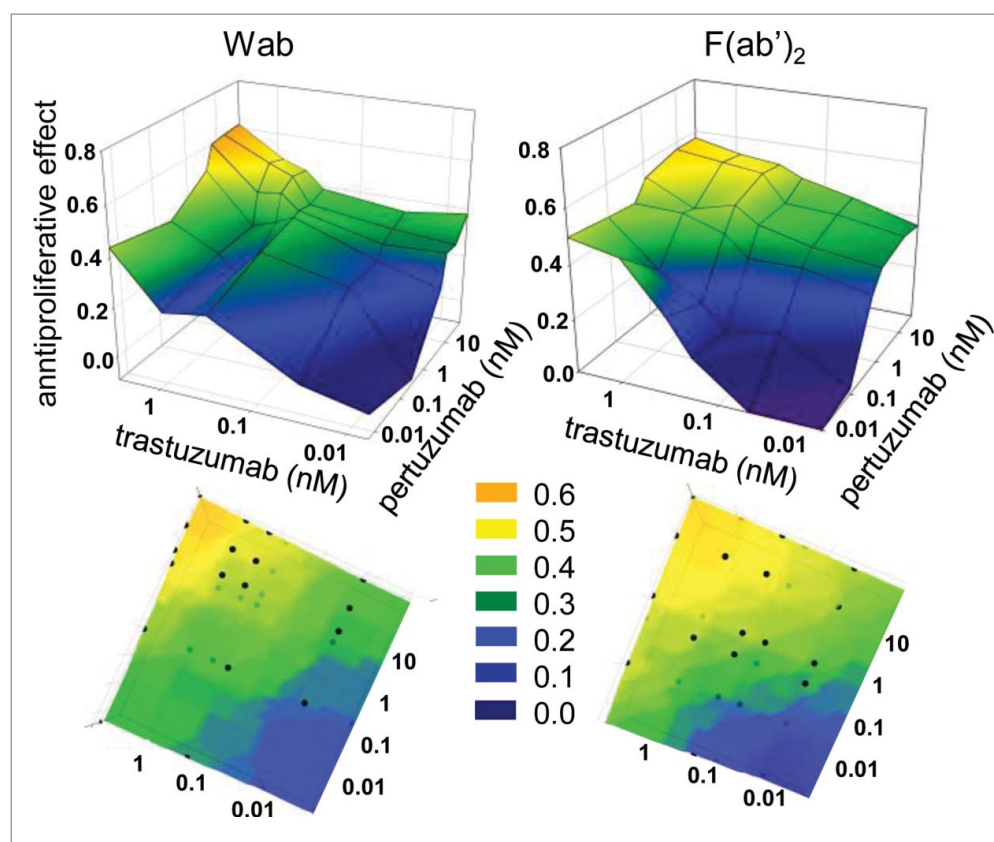
Consequently, we recommend that the 2 antibodies should be used in combination at the currently approved clinical dosage regimes to achieve maximum effect both in ADCC and in direct biological growth inhibition.

## Results

In order to distinguish between direct biological effects of the antibodies and their ability to recruit ADCC, F(ab')<sub>2</sub> of trastuzumab and pertuzumab were produced using pepsin digestion. Since the digested F(ab')<sub>2</sub> still has some affinity for protein A and protein G, the F(ab')<sub>2</sub> were purified using size-exclusion chromatography. The product was a homogeneous band in non-reducing SDS-PAGE, and bound with high affinity to native HER2 on cell surfaces, but did not bind anti-Fc



**Figure 1.** In vitro effect of anti-Her2 antibodies and F(ab')<sub>2</sub> on the proliferation of HER2+ tumors. Proliferation inhibition of the trastuzumab-sensitive BT-474 cell line (a,b) and the trastuzumab-resistant cell line JIMT-1 (c) was measured by an MTT-based colorimetric assay. All measured points are average ± SD of triplicates from ≥3 independent experiments normalized to untreated control. Half maximal effective inhibitory concentration (EC<sub>50</sub>) was calculated from the fitted dose-response curves.



**Figure 2.** Isobole analysis of in vitro biological growth inhibitory effect of trastuzumab and pertuzumab combinations. Proliferation of BT-474 cells was assessed with MTT assay. Data are from  $\geq 3$  replicates from  $\geq 3$  independent experiments. The vertical axis of the 3D coordinate systems show how much the proliferation rate decreased compared to the untreated control. The x and y axes represent trastuzumab and pertuzumab concentrations, or that of their F(ab')<sub>2</sub> variants. Doses are centered around the EC<sub>50</sub> values determined in Fig. 1. Bottom images show the top-view, smoothed images of the corresponding upper figures.

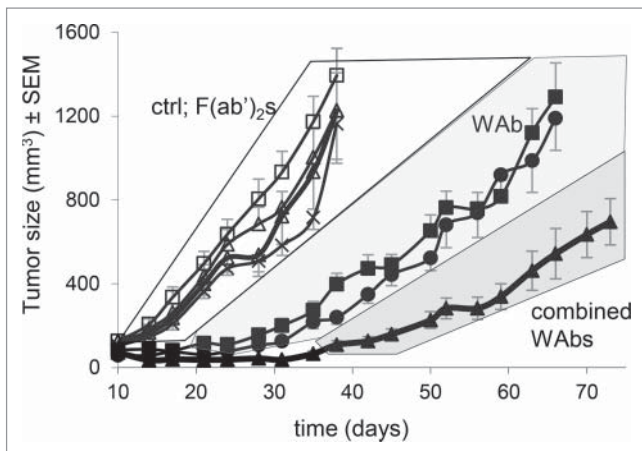
polyclonal antibodies (Expanded View Fig. 1), and did not mediate ADCC in vitro (Expanded View Fig. 2).

The in vitro effect of trastuzumab and pertuzumab, as well as their F(ab')<sub>2</sub>, on the proliferation of HER2-positive breast tumor cell lines was tested with an MTT-based colorimetric assay (Fig. 1). Both trastuzumab IgG and trastuzumab F(ab')<sub>2</sub> decreased the proliferation of the trastuzumab sensitive BT-474 cell line to a similar extent, with similar half maximal effective concentrations of 0.5–0.8 nM (SEM = 0.1, Fig. 1a). Pertuzumab IgG was less efficient in decreasing the proliferation of BT-474 (EC<sub>50</sub> = 7.2 nM, SEM = 2.2); however, the EC<sub>50</sub> of its F(ab')<sub>2</sub> was nearly 20-times smaller (EC<sub>50</sub> = 0.4 nM, SEM = 0.1) (Fig. 1b). This can be explained by its ability to more effectively disrupt the interactions of HER2 with other epidermal growth factor receptor (EGFR) family members owed to its smaller size.<sup>29</sup> The trastuzumab-resistant JIMT-1 cell line showed in vitro trastuzumab and pertuzumab resistance, and the removal of the Fc region did not alter this (Fig. 1c).

Next, isoboles were measured, also using MTT-based proliferation assay, to assess whether combining trastuzumab and pertuzumab IgGs, or their F(ab')<sub>2</sub> had an additive or synergistic effect on the proliferation of the sensitive BT-474 cells in vitro (Fig. 2). Doses were gradated in logarithmic steps symmetrically upwards and downwards of the half effective dose determined in Fig. 1. Combining either trastuzumab and pertuzumab IgGs, or their F(ab')<sub>2</sub>s, resulted in stronger inhibition of proliferation than using either whole antibody or F(ab')<sub>2</sub> alone. The isobole

bands corresponding to equal growth inhibitory effects (color coded for better viewing) run straight across between equivalent concentrations of the singly applied antibodies. This suggests that the combination of trastuzumab and pertuzumab exert an additive, but not a synergistic effect in decreasing cell proliferation in vitro, regardless of being present as whole antibodies or F(ab')<sub>2</sub>. In the case of the trastuzumab-resistant JIMT-1 cell line, none of the antibody combinations offered any in vitro proliferation inhibition (data not shown).

We therefore tested both trastuzumab and pertuzumab, as well as their F(ab')<sub>2</sub>, either alone, or in combination, for their ability to inhibit the growth of JIMT-1 tumor xenografts in SCID mice. Fig. 3 shows that both trastuzumab and pertuzumab whole antibodies inhibited tumor outgrowth for a period of a month after inoculation, similarly to our earlier findings. However, there was no conceivable difference between the efficacy of trastuzumab and pertuzumab. When the 2 antibodies were administered at the same dose, but together, tumor outgrowth was delayed compared to the effect of either antibody alone, and was significantly slowed throughout the experiment. At the same time, no effect on tumor growth was detected for either the F(ab')<sub>2</sub> or their combination compared to the solvent-treated control. This observation is coherent with JIMT-1 cells being in vitro resistant to the biochemical effects of trastuzumab and pertuzumab, and emphasizes the importance of ADCC during in vivo antibody therapy.

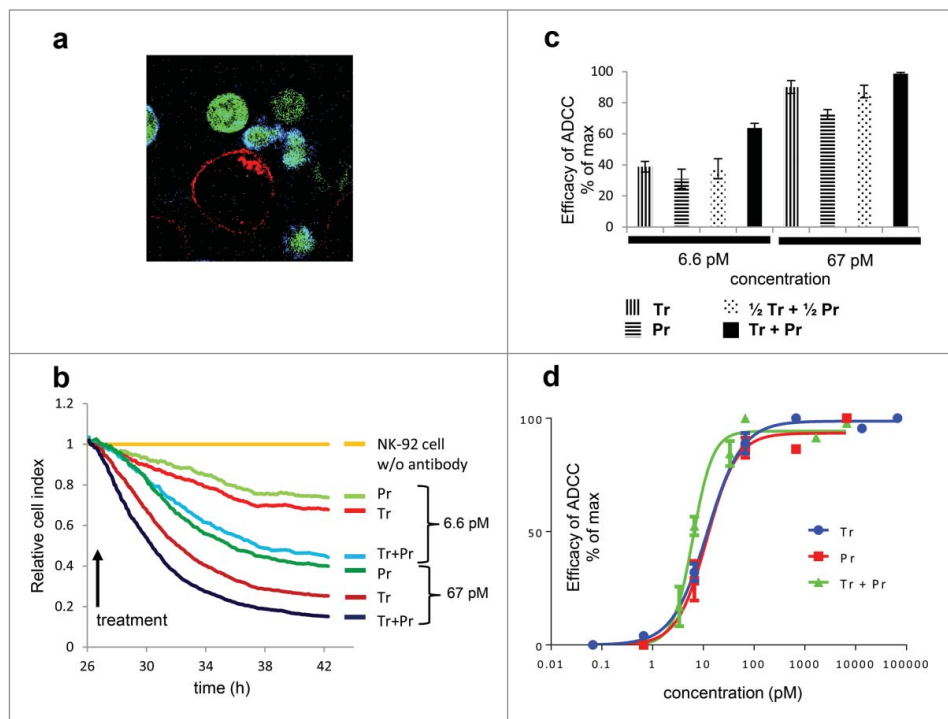


**Figure 3.** Effect of anti-HER2 antibodies and their  $F(ab')_2$  on in vivo tumor growth. Antiproliferative effect of the antibodies on JIMT-1 was examined in subcutaneously xenotransplanted SCID mice. Each group consisted of 6–8 mice. Compared to untreated control, neither trastuzumab  $F(ab')_2$ , pertuzumab  $F(ab')_2$ , nor their combination inhibited tumor growth in SCID mice. Trastuzumab or pertuzumab whole antibodies significantly reduced tumor growth compared to control and to all  $F(ab')_2$  treatments from day 21 onwards ( $p = 0.05$ ) and did not differ from each other significantly. Combination of the 2 whole antibodies achieved greater tumor growth inhibition, the difference from trastuzumab and pertuzumab alone was significant from day 38 and 42 onwards, respectively ( $p = 0.05$ ). Tumor sizes in the shaded areas (white, light gray, dark gray) significantly differed between the areas, but not within the areas ( $p < 0.05$ ).

To explore the mechanism behind improved ADCC mediated by the combination of trastuzumab and pertuzumab, we quantitatively assessed in vitro ADCC using an ECIS Z $\theta$  real time adherent cell analyzer. The CD16.176V.NK-92

immortalized effector cell line was used to make the ADCC measurements reproducible and independent of quality of donor peripheral blood mononuclear cells (PBMCs), which might fluctuate from experiment to experiment. To visually verify NK cell immune synapse formation at saturating antibody concentration, confocal microscopy of eGFP-tagged CD16.NK-92 cells targeting JIMT-1 cells was performed, after labeling CD16 on the NK cells and HER2 on the target cells (Fig. 4a). Next, we determined the range of antibody concentration where ADCC was evoked but was not saturated (Figs. 4b–d). At 6.6 pM, the antibody has already caused a considerable extent of cell killing, while this concentration applied without NK cells had no effect on cell proliferation (Figs. 1a and 1b). The extent of cell killing by 67 pM of either trastuzumab or pertuzumab was almost 90%, close to saturation (Figs. 4b, 4c), and concentrations above that caused maximum killing (Fig 4d).

In order to define how the combined effect of trastuzumab and pertuzumab relates to the ADCC evoked by their individual application, concentrations for single treatment were set to 6.6 pM and 67 pM, and compared to combinations using the same concentrations of the each antibody (Fig. 4b, 4c), as well as combinations using half of these concentrations, 3.3 pM and 33 pM for each antibody (Fig. 4c). The  $F(ab')_2$  were not studied extensively in this system because none of them decreased the cell index; neither alone nor in combination did they induce ADCC (Supplementary Fig. 2). Our data reveal that both trastuzumab and pertuzumab IgG antibodies induced ADCC, and thus decreased the cell index in a dose-dependent manner, pertuzumab being slightly less efficient. Using combination treatments where the total antibody concentration (3.3 pM + 3.3 pM, or 33 pM + 33 pM) was equal to



**Figure 4.** In vitro ADCC mediated by trastuzumab and pertuzumab. Confocal microscopy visualizes in vivo synapse formation induced by trastuzumab and pertuzumab. Red: HER2, green: eGFP expressing NK-92 cells, blue: CD16, FOV  $60 \mu\text{m} \times 60 \mu\text{m}$ . Quantitative, population level in vitro ADCC of JIMT-1 target cells with CD16.176V.NK-92 effector cell line was measured on ECIS Z $\theta$  real-time cell analyzer. Traces from one experiment are shown in (b). Effector/target cell ratio was 2.5:1 in all cases. Cell indices of antibody-free samples with NK-92 cells present were the same as double negative (NK-92 and antibody free) control and were used as reference for normalization. Reduction of cell number (impedance) at the end-point of each trace, averaged for  $\geq 2$  replicates per  $\geq 3$  independent experiments is shown in (c). Dose response curves fitted to the Hill equation are presented in (d).

the comparable single treatment (6.6 pM or 67 pM), we detected very similar degrees of cytotoxicity that were statistically identical. Also, for the nearly saturating concentrations, combination of the two antibodies, to reach twice the concentration of singly applied antibodies, could not significantly increase the efficacy of killing. However, for the non-saturating antibody concentrations, the combination yielding twice the concentration of single applications resulted in doubling the average efficacies of the single treatments (Fig. 4b, 4c). Accordingly, the  $EC_{50}$  value for combined treatment determined from Hill-plots (Fig. 4d) was 6.1 pM, as compared to 12.0 pM and 11.5 pM for trastuzumab- and pertuzumab-mediated ADCC, which suggests an additive effect.

To verify that such an additive effect could also exist in vivo, we quantitated the density of penetrating NK cells as a function of penetration depth in frozen sections of the tumors removed at the end of the in vivo experiment. NK cells were defined as 7–10  $\mu\text{m}$  CD45-positive, HER2-negative cells, containing unanimously identifiable DAPI stained nuclei. We imaged the central 10  $\mu\text{m}$  part of 14  $\mu\text{m}$  thick tissue sections divided into 3 confocal slices to detect and evaluate the small, moderately fluorescent murine NK cells. Images of vehicle-treated control and combined antibody-treated tumors are shown in Fig. 5a. HER2 of the tumor is shown in green, nuclei in blue, and CD45 on murine NK cells in red (or, when it overlaps along the z axis with the nucleus, purple). NK cells were counted and their density plotted as a function of penetration depth (Fig. 5b). NK cell

concentration was higher at the margins of the section, and decreased toward the center of the tumor. Both trastuzumab- and pertuzumab-treated tumors showed elevated numbers of NK cells compared to control. The tumors receiving combination treatment exhibited an even higher density of NK cells in the deeper areas. We also observed that NK cells could penetrate the tumor (stroma and matrix) deeper in the case of IgG combination treatment, creating longer and larger fissures along which they could attack antibody-binding tumor cells.

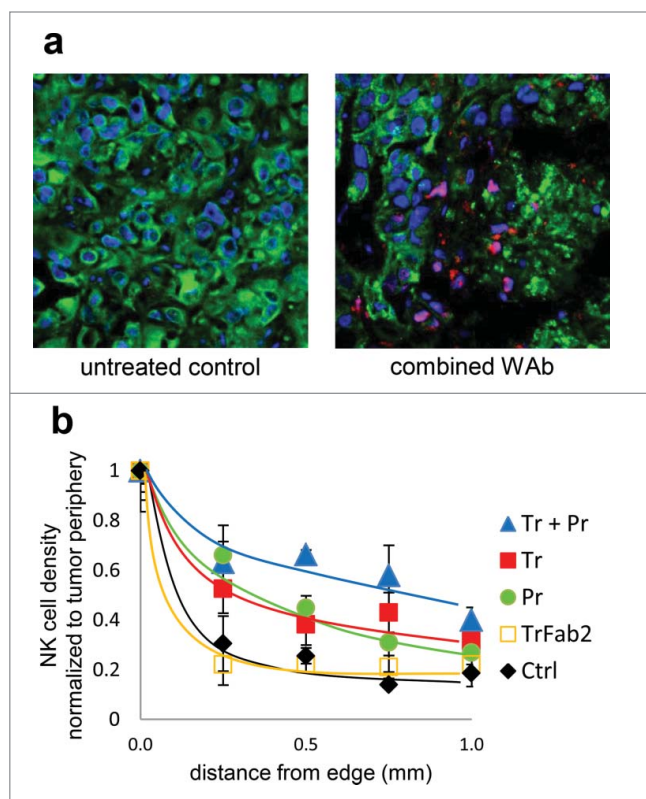
A closer inspection also revealed that NK cells belong to 2 distinct morphological groups: flattened NK cells embracing HER2-positive tumor cells are likely functionally active, while round NK cells distant from tumor cells are not engaged. In addition to the differences of absolute NK cell densities, it is interesting to note the ratio of flattened to round NK cells. This was 0.48 in  $F(ab')_2$  treated tumors, increased to 1.17 in single IgG treated ones, and was even higher, 1.68 in the case of tumors treated with antibody combination, suggesting that the NK cells in anti-HER2 IgG treated tumors were not only present in higher numbers, but also taking an active role.

## Discussion

This study was undertaken to examine if the combination of trastuzumab and pertuzumab provides any benefit in treating HER2-positive tumors that are in vitro resistant to trastuzumab, and to examine the mechanisms behind the possible beneficial effect. To distinguish between direct biological (signaling-based) effects of the antibodies and ADCC mediated by them in vivo, we generated  $F(ab')_2$  of both trastuzumab and pertuzumab. We verified that these  $F(ab')_2$  do not bind anti-Fc polyclonal antibodies, do not mediate ADCC in vitro, but exert equal or greater antiproliferative effect in vitro on the trastuzumab sensitive BT-474 cells. This model, reflecting the direct biological effect of the antibodies, also allowed us to study quantitatively the interaction between trastuzumab and pertuzumab when applied in combination. Isoboles revealed that the combination of the two whole antibodies, as well as that of their  $F(ab')_2$ , exerted an additive effect, with no evidence for synergism. The maximum effect of the combined administration of the two antibodies is higher than the maximum effect of either antibody alone. This reflects the distinct mechanism by which each of them affects HER2 signaling, downregulation of HER2 by trastuzumab<sup>8</sup> and inhibition of its dimerization with EGFR family members by pertuzumab,<sup>20</sup> which can independently inhibit cell growth.

While neither of the antibodies or their  $F(ab')_2$ , nor their combination had any direct biological effect on the JIMT-1 cells, the whole antibodies could mediate ADCC against these cells in vitro. This ADCC showed an additive effect for non-saturating concentrations of the antibodies; however, under saturating concentration of each antibody, the maximum ADCC did not increase when both antibodies were applied together. This is explained by the single mechanisms of ADCC, which can be made more efficacious by engaging twice as many  $Fc\gamma$  receptors on the same target, with two antibodies binding to different epitopes, but is saturated when enough  $Fc\gamma$  receptors are collected into the immune synapse of the NK cell.

In vivo growth of trastuzumab-resistant JIMT-1 tumors was not affected by the  $F(ab')_2$  of either trastuzumab, pertuzumab, or



**Figure 5.** NK cell infiltration of JIMT-1 tumor xenografts. Frozen sections of excised tumors were fluorescently labeled for HER2 (green), NK cells (CD45, red/magenta), and nucleic DNA (blue). Sections from an untreated control (left) and one treated with trastuzumab plus pertuzumab combination (right) are shown in (a). Treatment with antibodies enhances NK cell invasion, which results in extensive tumor lesion and a less dense tumor tissue structure. NK cell penetration into the tumor was quantitatively analyzed by counting CD45+ nucleated cells in 0.05 mm<sup>2</sup> areas starting from the side of the tumor toward the center (b).

their combination, consistent with the lack of their effect *in vitro* and their inability to recruit ADCC. Trastuzumab prevented the outgrowth of tumors as established earlier.<sup>12</sup> Also, pertuzumab exerted a similar antitumor effect. Combination of the two antibodies prolonged the tumor-free period and significantly slowed tumor growth. This implies that ADCC is a valuable therapeutic mechanism of action in the case of *in vitro* trastuzumab-resistant HER2-positive tumors, and that combining two distinct monoclonal antibodies, which can both mediate ADCC against the same molecular target, can be highly beneficial. In fact, we observed that the combination treatment increased the number of invading NK cells, as well as the proportion of NK cells actively engaged in killing, and this enhanced recruitment negatively correlated with tumor progression and size.

To analyze how the combination of the two antibodies enhances ADCC, we measured ADCC efficacy *in vitro* as well, as a function of antibody concentration. Using the same JIMT-1 cells as targets, we found that ADCC was saturable with increasing doses, and the combination of the 2 antibodies enhanced ADCC additively only in non-saturating concentrations. Since the same combination *in vivo* also produced an antitumor effect that was at least additive, it is likely that *in vivo* doses used in these experiments do not saturate ADCC either. One obvious possibility is that the *in vivo* administered antibody doses of 5 mg/kg resulted in non-saturating tissue concentrations in the tumor.<sup>30</sup> Although it is highly challenging to give a correct estimate of antibody tissue levels,<sup>30,31</sup> since the doses are comparable to those in clinical application but administration in mice was done even more frequently (twice per week) than the clinical administration (bi-weekly or even less frequently), tissue levels in the clinical setting might even be lower than those we achieved in mice.

Other factors to consider are the lower NK cell to target ratios *in vivo*, and the lower affinity FcγRIII receptors on native NK cells compared to those in CD16.NK92 cells. These factors together can all contribute to a decreased saturation of ADCC *in vivo*, which implies that if the two antibodies are applied in combination, each at their usual therapeutic dose, *in vivo* ADCC can be additively enhanced.

From these observations, we can conclude that ADCC mediated by anti-HER2 antibodies is able to efficiently combat even those HER2-positive tumors that are resistant to trastuzumab, which in the case of breast cancer can be over half of the cases.<sup>6,7</sup> Since both ADCC and the direct biological effects are additive at the maximum tolerated/clinically permitted doses, combination of trastuzumab and pertuzumab is the best choice for all HER2-positive tumors. Specifically, if the tumor is trastuzumab resistant, the higher total tissue concentration of the antibodies, and possibly their sterically distinct attachment sites can improve the efficacy of the patient's own NK cells. If the tumor is trastuzumab sensitive, the antitumor effects of the distinct molecular mechanisms are also added to the effect of ADCC. These two additive mechanisms underpin the findings of the clinical biomarker analysis conducted during the CLEOPATRA trial, which revealed that "HER2 is the only marker suited for patient selection" and "only HER2, HER3, and PIK3CA were relevant prognostic factors."<sup>32</sup>

Once the principle of additively applying the maximum tolerable doses has been established, it seems evident to look for additional agents with similar molecular targeting specificity. In the

case of HER2-positive tumors, the dual (HER2 and EGFR) tyrosine kinase inhibitor lapatinib has successfully been applied in combination with chemotherapy and approved by the Food and Drug Administration (FDA).<sup>33</sup> It has recently (March 2016) been announced at the Official 10th European Breast Cancer Conference that in the EPHOS-B randomized trial, the combination of lapatinib and trastuzumab shrunk HER2-positive breast cancer significantly in 11 d after diagnosis, providing an advantage over trastuzumab alone: pathologically complete response was observed in 11% vs 0%, and minimal residual disease in 17% vs 3% of the patients.<sup>34</sup> The beneficial effect of the combination is probably based on providing an alternative blocking of HER2 (and EGFR) function through kinase inhibition also in those cells that do not bind trastuzumab well, or do not respond with a downregulation of HER2. This complementarity appears to be also true for the reverse situation, wherein addition of trastuzumab to lapatinib monotherapy was found to improve median overall survival by 4.5 months.<sup>35</sup> In light of this, the triple combination of lapatinib, trastuzumab and pertuzumab could be even more efficacious than combining trastuzumab and pertuzumab, although the tolerability of kinase inhibition in the long run, especially that of EGFR, could be an issue.<sup>36</sup>

Overall, it appears safe and highly beneficial to use combination antibody therapy against HER2-positive tumors from the start, which is currently being tested in the Phase 3 APHINITY clinical trial. Switching to combination in the case of ongoing trastuzumab monotherapy is also supported both by clinical and preclinical data. On the one hand, the combined use of trastuzumab and pertuzumab was beneficial in cases where the tumor has progressed following trastuzumab monotherapy.<sup>24</sup> On the other hand, we observed earlier that circulating and disseminated tumor cells are still sensitive to ADCC when the primary tumor has already become resistant,<sup>19</sup> probably owed to their lack of a well-developed ECM.<sup>13,18</sup> Consequently, ADCC enhanced by combination antibody therapy could better prevent the dissemination of tumor cells even when the primary tumor or larger inoperable metastases become resistant to the combination. Swain et al.<sup>27</sup> conclude their account of the CLEOPATRA trial by stating: "Finally, the question of when, if ever, therapy with pertuzumab plus trastuzumab for metastatic breast cancer should be stopped remains unanswered." Considering the additive effect of this combined treatment in mediating ADCC and the importance of ADCC in preventing tumor dissemination, including that from previously dormant tumor cells, we propose that in the adjuvant setting combination antibody therapy of HER2-positive breast cancer should be the first choice, and possibly it should not be stopped.

Collectively, in any clinical setting the combination of both antibodies at maximum clinically approved doses should be applied to recruit maximum ADCC and cause maximum direct biological growth inhibition. This is the mechanistic background, based on which the combined therapy is expected to give results that are superior to monotherapy, whatever the type of HER2-positive tumor may be.

## Materials and methods

All materials were from Sigma-Aldrich (St. Louis, MO), unless otherwise indicated.

## Cells

**BT-474 (HTB-20<sup>TM</sup>)** and **SKBR-3 (HTB-30<sup>TM</sup>)** cells derived from trastuzumab-sensitive, HER2-positive invasive ductal adenocarcinomas were obtained from the American Type Culture Collection. The cells were grown in RPMI (R6504) that was supplemented with 20% FCS, insulin, glutamine and antibiotics and in DMEM (D7777) that was supplemented with 10% FCS (F9665), glutamine and antibiotics in a 5% CO<sub>2</sub> humidified incubator with glutamine antibiotics and passaged 3 times weekly. **JIMT-1** is a HER2-positive, trastuzumab-resistant human breast cancer cell line, established from pleural metastasis in the laboratory of Cancer Biology, University of Tampere, Finland.<sup>37</sup> JIMT-1 cells were cultured in 1:1 ratio of Ham's F-12 (N6760) and DMEM supplemented with 20% FCS, 300 U/L insulin (I9278), glutamine and antibiotics and split every 3–4 d. For in vitro ADCC, the cell line **CD16.176V.NK-92**, here abbreviated NK92 (from Dr. Kerry S. Campbell, the Fox Chase Cancer Center, Philadelphia, PA) was used. These cells are derived from a human NK-like phenotype non-Hodgkin's lymphoma,<sup>38</sup> and have been transduced to express a high affinity variant (176V) of FcγRIIIA receptor (CD16).<sup>39,40</sup> A variant of this cell line co-expresses eGFP with CD16. The cell lines were cultured in special NK medium of α-MEM (M0644) containing 10% FCS and 10% horse serum (Hyclone SH 30070), supplemented with glutamine, non-essential amino acids, Na-pyruvate (S8636), antibiotics and IL-2 at 100 IU/ml (Proleukin 18 × 10<sup>6</sup> NE, Novartis). All cell cultures were routinely checked for the absence of mycoplasma contamination.

## F(ab')<sub>2</sub> preparation from whole immunoglobulins

Trastuzumab (Herceptin<sup>®</sup>, Roche) and pertuzumab (Perjeta<sup>®</sup>, Roche) antibodies were dissolved in 20 mM acetate buffer (pH 4.5), dialyzed thrice against the same buffer and concentrated using Amikon Ultra filters (Millipore, UFC 801096.). The Fc domain was digested by immobilized pepsin (ThermoScientific, 20343). The reaction was stopped by 10 ml 2 M TRIS-HCl, pH 8.2. Immobilized pepsin was cleared by 5 min centrifugation at 300 xg, and the supernatant was filtered sterile and concentrated on Amikon Ultra filters. Sephacryl S300 and Superdex 200 (GE Health Care Life Science, 17-0438-09 and 17-1043-99) gel filtration columns were used for size-exclusion chromatography purification of trastuzumab F(ab')<sub>2</sub> and pertuzumab F(ab')<sub>2</sub>, respectively. Absorbance of fractions was measured with a NanoDrop ND 1000 (Thermo Science), protein content was assessed with non-reducing SDS-PAGE, and fractions contaminated with smaller fragments and partially digested or undigested antibodies were pooled and re-chromatographed. F(ab')<sub>2</sub> were tested for lack of the Fc, for preserved HER2 binding affinity and for inability to mediate ADCC (see supplementary data).

## Flow cytometric assessment of F(ab')<sub>2</sub> quality

SKBR-3 cells were grown in DMEM supplemented with 10% FCS in a 5% CO<sub>2</sub> humidified incubator with antibiotics and passaged twice weekly. Freshly detached SKBR-3 cells were treated with trastuzumab, pertuzumab, or their F(ab')<sub>2</sub> at

10 μg/ml concentration for 10 min at RT, then labeled with Alexa Fluor 488 conjugated goat-anti-human Fc or Alexa Fluor 488 (A11013) conjugated goat anti-human (H<sup>+</sup>L) antibodies at 10 μg/ml for 10 min. HER2-positivity of SKBR-3 cells was verified with Alexa Fluor 647 conjugated 76.5 antibody. Measurements were performed on a BD FACSCalibur flow cytometer, 20,000 events were collected and the median of histograms is displayed.

## In vitro cell proliferation

The effect of the antibodies on in vitro proliferation of HER2-expressing, trastuzumab sensitive and resistant cell lines were tested using an MTT-based colorimetric cell proliferation assay (EZ4U, Biomedica GmbH, BI-5000). Cells were incubated in 96-well plates for 3 d in the presence or absence of antibodies or their F(ab')<sub>2</sub> at the given concentrations. After incubation with EZ4U, absorption (λ = 488 nm, corrected with absorption at λ = 620 nm) of the metabolic substrate was measured on Synergy HT Multi-Detection microplate Reader (BioTek). Replicates (n ≥ 3) were averaged, and growth inhibition was expressed as normalized to untreated control.

## In vitro ADCC

Inability of the F(ab')<sub>2</sub> to recruit ADCC was tested with PBMC as effectors using the xCELLigence (Roche) real time cell analyzer. Cell index was calculated by the analyzer based on the measured impedance. BT-474 cells were seeded in 8-well chambers (Applied Bioscience, 8W10E) with gold electrodes and allowed to proliferate until 80% confluence (50 hours). Mononuclear leukocytes freshly separated from human PBMC were added at a 5:1 ratio to the BT-474 cells and impedance was monitored for 24 h. Data from 3 wells were averaged and traces normalized to impedance measured at the start of ADCC.

For quantitative analysis of ADCC efficiency in vitro, an ECIS Z<sup>θ</sup> real-time cell analyzer was used. JIMT-1 target cells were grown in 8W10E PET 8well arrays with gold electrodes at the bottom. The complex impedance spectrum of cells adhered to the electrodes was assessed in a the range of 1 Hz to 100,000 Hz. Effector/target ratio was set at 2.5:1, considering that NK cells constitute around 15% of the freshly separated PBMC, and both in our previous experiments and in the literature a 15:1 PBMC to target cell ratio was determined to be highly efficient. NK92 effector cells or antibodies at defined concentration were administered after impedance of the target cells has reached a plateau.

## Xenograft tumors and in vivo antibody treatment

SCID (C.B-17/Icr-Prkdc<sup>skid</sup>/IcrIcoCrI, Fox-Chase) mice were purchased from Charles River Laboratories, and housed in a specific-pathogen-free environment. All animal experiments were performed in accordance with FELASA guidelines and recommendations and DIN EN ISO 9001 standards. Only non-leaky SCID mice with murine IgG levels below 100 ng/ml were used. Each seven-week-old female SCID mouse participating in the study was given a subcutaneous injection of 5 × 10<sup>6</sup> JIMT-1 cells suspended in 100 μl Hanks' A buffer and mixed with an



equal volume of Matrigel (BD Matrigel, BD Biosciences, 356237) in both flanks. Tumor volumes were derived as the product of the length, width and height of the tumor measured 2 times a week with a caliper. Trastuzumab ( $n = 8$ ), trastuzumab F(ab')<sub>2</sub> ( $n = 7$ ), pertuzumab ( $n = 8$ ), pertuzumab F(ab')<sub>2</sub> ( $n = 7$ ), trastuzumab+pertuzumab ( $n = 8$ ), trastuzumab F(ab')<sub>2</sub> + pertuzumab F(ab')<sub>2</sub> ( $n = 7$ ), and, as a control, HEPES buffer ( $n = 7$ ) was injected intraperitoneally in a volume of 100  $\mu$ l HEPES buffer twice weekly. The injected dose was 100  $\mu$ g of each antibody or F(ab')<sub>2</sub> used. Treatment commenced at the time of JIMT-1 inoculation and was continued until the end of the experiment. At the end of the experiment the animals were euthanized by cervical dislocation. The experiments were done with the approval of the National Ethical Committee for Animal Research (# 4/2012/DE MÁB).

### ***In vitro* immune synapse formation by CD16.NK-92 Cells**

JIMT-1 cells were seeded in Poly-L-Lysine (81804) coated 2 × 9 well  $\mu$ -slides (Ibidi) at 50,000 cells/cm<sup>2</sup> density and incubated for 2 d in JIMT-1 medium. After that, cells were treated with trastuzumab and pertuzumab whole antibodies at 10  $\mu$ g/ml concentration in 20  $\mu$ l indicator-free NK medium at 37°C, 5% CO<sub>2</sub>, for 10 minutes. After 10 minutes, 120,000 NK92 eGFP cells/well were added in 20  $\mu$ l medium and cocultured at 37°C, 5% CO<sub>2</sub>, for 10 minutes. Fluorescently labeled antibodies were added at 10  $\mu$ g/ml final concentration; CD16 was labeled with Alexa Fluor 647 conjugated anti-CD16 antibody (clone 3G8, BD PharMingen, 302023), HER2 was labeled with an Alexa Fluor 555 conjugated Fab, clone 76.5.

### ***Tumor xenograft sections***

At termination, mice were dissected and fresh tumors were embedded in cryomatrix (Thermo Fischer Scientific, 6769006) and snap frozen in liquid nitrogen. Serial 14  $\mu$ m thick cryosections were made with a Shandon Cryotome (Thermo Fischer Scientific) at -24°C and air-dried. Labeling was carried out at room temperature and all antibodies were diluted in HEPES buffer supplemented with 1% BSA (A7906). After 5 min of rehydration in HEPES buffer containing 1% BSA and 0.01% TritonX-100 (Thermo Fischer Scientific, 28314) HER2 was labeled with Alexa Fluor 488 conjugated 76.5 antibody, NK cells were labeled with Alexa Fluor 647 conjugated anti-CD45 (clone CD45.1). Both antibodies were used at 2  $\mu$ g/ml concentration at 4°C for 10 hours. Nuclei were stained with DAPI (D9564) at 10  $\mu$ g/ml for 2 hours. Sections were washed for 5, 20, and 60 minutes, fixed in formaldehyde, and mounted in Mowiol (Sigma, 81381) antifade.

### ***Confocal laser scanning microscopy***

Fluorescence-labeled cells and tissue sections were analyzed with confocal laser scanning microscope (LSM 510, Carl Zeiss GmbH) using a 40× C-Apochromat water immersion objective (NA = 1.2). DAPI was excited at 351 nm, Alexa Fluor 488 and eGFP were excited at 488 nm, Alexa Fluor 546 and Alexa Fluor 555 at 543 nm, and Alexa Fluor 647 at 633 nm. Corresponding fluorescence emission was separated with an appropriate quad-

band dichroic mirror, and detected through 385 to 470 nm, 505 to 550 nm, 560 to 615 nm bandpass and 650 nm longpass filters, respectively. For tissue samples, 3 consecutive, 4  $\mu$ m thick optical sections were taken at 3  $\mu$ m intervals, covering the central 10  $\mu$ m part of the sections. For detecting immune synapses between NK cells and target cells, 1.5  $\mu$ m optical sections were used.

### ***Statistical evaluation of data***

To analyze dose effect curves, data were fitted with the Hill equation. For comparison of the efficacy of ADCC evoked by trastuzumab, pertuzumab and their combination, data points in each experiment were normalized to the maximum effect reached with saturating concentrations, and the mean for each treatment and concentration was normalized to the overall maximum effect. *In vivo* tumor sizes at each sampling time point were compared with one-way ANOVA followed by Tukey's multiple comparison test, at  $\alpha = 0.05$ . SigmaPlot 12 and GraphPad Prism 6 were used for analysis.

### ***Disclosure of potential conflicts of interest***

No potential conflicts of interest were disclosed.

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### ***Authors' contributions***

GT carried out *in vitro*, *in vivo* and *ex vivo* experiments, performed data analysis and wrote the manuscript, ÁS participated in study design, performed data analysis, and wrote the manuscript, LS carried out and evaluated *in vivo* experiments and wrote the manuscript, YY conceived the project, participated in study design and wrote the manuscript, JS participated in study design, analyzed results and wrote the manuscript, GV conceived and coordinated the study, designed experiments, carried out *in vitro* experiments, analyzed data, and wrote the manuscript. All authors have read and approved the final manuscript.

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